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Glial-Restricted Precursors Are Derived from Multipotent Neuroepithelial Stem Cells[1]

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Neuroepithelial cells in the developing ventricular zone differentiate into neurons, astrocytes, and oligodendrocytes. It is not known, however, whether this differentiation occurs in a single step or is a pathway utilizing intermediate more restricted precursor cells. To characterize the generation of glial cells from multipotent stem cells we have cultured neur epithelial (NEP) cells from E10.5 rat embryos. Cultured NEP cells do not express any glial differentiation markers when grown on fibronectin/laminin under nondifferentiation conditions. NEP cells, however, differentiate into A2B5 immunoreactive cells which can subsequently give rise to oligodendrocytes and astrocytes. Clonal analysis of NEP cells dem nstrates that the A2B5 immunoreactive cells arise in clones that contain neurons and astrocytes, indicating that A2B5$^+$ cells arise from multipotent NEP precursor cells. A2B5$^-$ cells, maintained as undifferentiated cells over multiple passages, can subsequently give rise to both oligodendrocytes and astrocytes. A2B5$^+$ cells, however, do not generate neurons. Thus A2B5$^+$ cells represent a restricted progenitor cell population that differentiates from a multipotent NEP cell. Based n our results we propose that differentiation of the multipotential NEP cells to terminally differentiated glial cells occurs via intermediate restricted precursors. © 1997 Academic Press

INTRODUCTION

Multipotent cells with the characteristics of stem cells have been identified in several regions of the central nervous system and at several developmental stages (for review see Gage *et al.*, 1995; Marvin and McKay, 1992; Skoff, 1996). These cells, often referred to as neuroepithelial stem cells (NEP cells), have the capacity to undergo self renewal and to differentiate into neurons, oligodendrocytes, and astrocytes (Davis and Temple, 1994; Gritti *et al.*, 1996; Reynolds *et al.*, 1992; Reynolds and Weiss, 1996; Williams *et al.*, 1991). The nervous system also contains prccursor cells with restricted differentiation potentials (Kilpatrick *et al.*, 1995; Price *et al.*, 1987, 1991; Reynolds *et al.*, 1992; Reynolds and Weiss, 1996; Williams, 1995; Williams *et al.*, 1991). Although it is likely that lineage-restricted cells are the progeny of multipotent cells, previous studies have not demonstrated a direct relationship between these two classes of cells (see reviews, Morrison *et al.*, 1997; Stemple and Mahanthappa, 1997; Temple and Qian, 1996).

To demonstrate a relationship between multipotent stem cells and more restricted precursors we analyzed the development of glial-restricted precursors from NEP cells. We chose to focus on the transition of multipotent NEP cells to glial precursor cells, as glial precursor cells are the best characterized intermediate precursor cells in the CNS. The most extensively studied precursor is called the oligodendrocyte-type-2 astrocyte progenitor (O-2A) (Noble *et al.*, 1990; Raff *et al.*, 1983; Tempel and Raff, 1986; Temple and Raff, 1985). O-2A precursors can be isolated and purified from postnatal rat optic nerve, cortex, and spinal cord and differentiate into postmitotic GalC$^+$ oligodendrocytes or into A2B5$^+$/GFAP$^+$ type-2 astrocytes, which are distinct from GFAP$^+$/A2B5$^-$ type-1 astrocytes (Raff *et al.*, 1983), depending on the culture conditions. O-2A progenitor cells exhibit a proliferate response to a variety of mitogens, such as platelet-derived growth factor (PDGF) and basic fibroblast

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growth factor (bFGF) (Gard and Pfeiffer, 1990; Mayer *et al.*, 1993; McKinnon *et al.*, 1990; Noble *et al.*, 1988) and can be kept in an undifferentiated dividing stage as long as both PDGF and bFGF are present in the culture medium (Bogler *et al.*, 1990). We have used antigenic markers and culture conditions described in the O-2A system to determine glial generation in the early spinal cord.

NEP cells derived from the embryonic spinal cord can be maintained in an undifferentiated stage over several passages but retain the ability to generate neurons, oligodendrocyte, and astrocytes (Rao *et al.*, (1996) *Neuroscience* Abstract 215.12). We show that NEP cells differentiate into A2B5 immunoreactive cells that lack markers for other cell lineages and have a morphology similar to that of O-2A progenitor cells isolated from other embryonic brain regions (Aloisi *et al.*, 1992; Fok-Seang and Miller, 1994; Temple and Raff, 1985). We provide evidence that these A2B5$^+$ cells arise from multipotent NEP cells, can be expanded for multiple passages without losing their multipotentality, and can generate oligodendrocytes and astrocytes but not neurons. NEP-derived A2B5$^+$ cells thus represent glial-restricted intermediate precursor cells. Using this system, we are able to demonstrate for the first time that cells can progress from a multipotential stage to a terminal differentiated end stage via intermediate lineage-restricted precursor cells.

MATERIALS AND METHODS

Substrate Preparation

Laminin, used at a concentration of 20 μg/ml (Biomedical Technologies Inc.), was dissolved in distilled water and applied to tissue culture plates (Falcon). For the fibronectin solution, fibronectin (Sigma) was resuspended to a stock concentration of 10 mg/ml and stored at −80°C and diluted to a concentration of 250 μg/ml in DPBS (Gibco). The fibronectin solution was applied to tissue culture dishes and immediately withdrawn. Subsequently the laminin solution was applied and plates were incubated for 5 hr. Excess laminin was withdrawn and the plates were allowed to air dry. Plates were rinsed with water and then allowed to dry again.

Neuroepithelial Cell Cultures

Sprague–Dawley rat embryos were removed at Embryonic Day 10.5 (13–22 somites) and placed in a petri dish containing Hanks balanced salt solutions (HBSS, Gibco). The trunk segments of the embryos (last 10 somites) were dissected using tungsten needles, rinsed, and then transferred to fresh HBSS. Trunk segments were incubated at 4°C in 1% trypsin solution (Gibco) for a period of 10 to 12 min. The trypsin solution was replaced with fresh HBSS containing 10% fetal bovine serum (FBS). The segments were gently triturated with a Pasteur pipette to release neural tubes free from surrounding somites and connective tissue. Isolated neural tubes were transferred to a 0.05% trypsin/EDTA solution (Gibco) and incubated for 10 min. Cells were dissociated by trituration and plated in 35-mm dishes (Nunc) at high density. Cells were maintained at 37°C in 5% $C0_2$/95% air. Cells were replated at low density after 3 days. Cells from several dishes were then harvested by trypsinization (0.05% Trypsin/EDTA solution for 2 min), pelleted, resuspended in a small volume, and replated at a density of 5000 cells/35-mm dish. The basal medium used in all experiments was a chemically defined medium modified from that described by Stemple *et al.* (1988). The medium consisted of DMEM-F12 (Gibco) supplemented with additives described by Bottenstein and Sato (1979) and bFGF (20 ng/ml) and CEE extract (10%) prepared as described previously (Stemple and Anderson, 1992).

Clonal Cultures of Neuroepithelial Cells

Cells were trypsinized and plated in 35-mm dishes coated with fibronectin/laminin at a dilution of 50 cells/dish. In some experiments cells were plated at 10 cells/dish. Cells were allowed to settle for a period of 4 hr, single cells were circled, and their development was followed in culture. In most experiments clonal cultures were terminated after 12 days. In experiments to demonstrate oligodendrocyte development clones were followed for 18–21 days. In these assays approximately 20–40% of single cells died within 24 hr. Of the remaining cells (60–80%) the large majority (< 90%) generated multipotent clones. Clonal plates were usually triple-labeled with the cell surface antigen and the appropriate secondary antibodies.

Generation of Neurons, Oligodendrocytes, and Astrocytes

Neuroepithelial cells cultured in nondifferentiating conditions for a period of 5 days were harvested by trypsinization and replated onto dishes sequentially coated with fibronectin/laminin (0.25 mg/ml) in neuroepithelial culture medium. For neuronal and oligodendrocyte differentiation, the medium consisted of neuroepithelial culture medium described above with the omission of 10% CEE (-CEE condition). For astrocyte differentiation the -CEE medium was supplemented with 10% fetal calf serum. Differentiation was assayed 5 days or 9 days after replating (as detailed in the results).

Immunopanning of A2B5$^+$ Cells

NEP cells were cultured in -CEE conditions for 6 days and the A2B5$^+$ cell population was purified using a specific antibody-capture assay (Wysocki and Sato, 1978) with modification utilized previously (Mayer *et al.*, 1994). In brief, cells were trypsinized and the suspension was plated on an A2B5 antibody (Eisenbarth *et al.*, 1979)-coated dish to allow binding of all A2B5$^+$ cells to the plate. The supernatant was removed and the plate was washed with DMEM supplemented with additives described by Bottenstein and Sato (1979) (DMEM-BS). The bound cells were scraped off and plated on fibronectin/laminin-coated glass coverslips in 300 μl DMEM-BS ± growth factors at 5000 cells/well. In the final culture the contaminating number of A2B5$^-$ cells represented less than 10% of the total cells. The A2B5 antibody for coating the plates was used at a concentration of 5 μg/ml protein. Cells were allowed to bind to the plate for 20–30 min in a 37°C incubator. Growth factors were added every other day at a concentration of 10 ng/ml. Recombinant human PDGF-AA was a kind gift from C. George-Nascimento and L. Coussens (Chiron Corp.). Recombinant rat CNTF was obtained from Precision Research Biochemicals. Recombinant bFGF was purchased from PeroTech Inc. and retinoic acid (RA) was obtained from Sigma.

Clonal Cultures of A2B5$^+$ Cells

After CEE withdrawal cells were stained with A2B5 and IgM-monoclonal antibody FITC (Southern Biotechnologies) and plated at a limited dilution in 96-well plates coated with fibronectin/laminin at a dilution of 1 cell/well. Cells were allowed to settle for a period of 4 hr and wells containing one A2B5$^+$ stained cell were recorded. Cells were cultured in the presence of PDGF and bFGF for 7 days in which clones usually reached a size of 50–200 cells/clone. After washing with bFGF-free DMEM-BS individual clones were grown in a medium supplemented with PDGF. In most experiments clonal cultures were stained after 12 days. In experiments designed to demonstrate oligodendrocyte development clones were followed for 18–21 days. In experiments where self-renewal capacity was demonstrated, clones were replated into 35-mm dishes and further expanded with PDGF/bFGF. Clones were propagated through four passages. After each passage clones were stained with A2B5 to determine their homogeneity.

Immunocytochemistry

Staining procedures were as described previously (Mayer *et al.*, 1994). The antibodies used are listed in Table 1. Staining for the cell surface markers A2B5, α-GalC, O4 (cell lines obtained from ATCC), and p75 (Yokoyama *et al.*, 1993) was carried out in cultures of living cells. To stain cells with antibodies against internal antigens like GFAP (Sigma), which recognizes specifically astrocytes (Bignami *et al.*, 1972), β-III tubulin (DAKO) and RT-97, which stain neurons (Geisert and Frankfurter, 1989), nestin, a marker for undifferentiated stem cells (Lendahl *et al.*, 1990), or 5-bromodeoxyuridine (BrdU, Sigma), to determine the number of dividing cells, cultures were fixed with ice-cold methanol. All secondary monoclonal antibodies were purchased from Southern Biotechnology.

Double labeling experiments were performed by simultaneously incubating cells in appropriate combinations of primary antibodies followed by non-cross-reactive secondary antibodies. In triple label experiments, cultures were incubated with the primary antibody in blocking buffer for a period of 1 hr, rinsed with buffer (PBS), and incubated with a species-specific secondary antibody in blocking buffer for 1 hr. Cultures were rinsed three times with PBS and examined under a fluorescent microscope. For labeling cultures with four antibodies simultaneously, live cells were first incubated with the surface antibodies A2B5 and α-GalC without the secondary layers. Clones were then fixed in ice-cold methanol for 10 min and stained with α-β-III tubulin and the appropriate secondary antibody. After scoring the result of this staining, which one of it usually being negative, clones were stained with GFAP and the secondary layer for the first set of surface antibodies. Finally, the secondary antibody for GFAP was added. This procedure allowed staining with four antibodies using only three fluorescent-color conjugated secondary antibodies.

RESULTS

Neuroepithelial Cells Do Not Express Oligodendrocyte Lineage Markers in Vivo and in Vitro

Neurons, oligodendrocyte, and astrocytes can be identified using a variety of lineage-specific markers (Table 1) (Eisenbarth *et al.*, 1979; Geisert and Frankfurter, 1989; Sommer and Schachner, 1981; Trimmer *et al.*, 1991). In addition to defining differentiated cells, some precursor cells can also be recognized by specific antibodies. We employed two such markers in this study: nestin is expressed by a variety of undifferentiated cells in the CNS (Dahlstrand *et al.*, 1992a, 1992b; Lendahl *et al.*, 1990; Tohyama *et al.*, 1992, 1993; Zimmerman *et al.*, 1994), while the A2B5 antibody labels O-2A progenitor cells (for summary see Table 1).

NEP cells *in vivo* do not label with any lineage markers tested (Fig. 1, α-GalC and α-β-III tubulin not shown) as determined by labeling the neural tube sections of rat embryos at 10.5 days gestation with differentiation markers (Fig. 1 and Table 1). Nestin was, however, expressed uniformly throughout the tissue.

To determine whether NEP cells isolated from the neural tube at that stage remained negative for differentiation markers *in vitro*, dissociated NEP cells from the rat spinal cord at Day 10.5 were plated on fibronectin/laminin coated dishes. Cells were cultured in the presence of bFGF (10 ng/ml) and 10% chick extract (CEE) for 3 days. In this culture condition, cells grew as a population of undifferentiated precursor cells which can be propagated in culture for at least 5 passages as long as bFGF and CEE are constantly supplied (Rao *et al.* (1996) *Neuroscience* Abstract 215.12). NEP cell cultures were established and stained after 3 days of *in vitro* growth with antibodies present on lineage committed glial cells (Table 1). Consistent with the *in vivo* staining pattern, cultures were α-nestin$^+$ and α-GFAP$^-$ and A2B5$^-$ (Fig. 2) as well as negative for other lineage markers (Table 1).

A2B5 Immunoreactivity Defines Glial-Specific Cells in the NEP Cell Population

A2B5 immunoreactivity identifies a glial precursor cell at various stages of development in the brain (Noll and Miller, 1993; Pringle and Richardson, 1993; Raff *et al.*, 1983; Warf *et al.*, 1991; Yu *et al.*, 1994) and spinal cord (Fok-Seang and Miller, 1994). We therefore examined the expression of A2B5 in NEP cultures. Seventy percent of NEP cells cultured in the absence of CEE for 3 days exhibit A2B5 immunoreactivity (data not shown, see, however, Fig. 3). These A2B5$^+$ cells had a flat morphology and were able to divide in the presence of bFGF. After 4 days in culture in the absence of CEE, 81 ± 7% of A2B5$^+$ NEP cells, stained with anti-BrDU for 24 hr, were engaged in cell division (Fig. 3). Double labeling the NEP-derived A2B5$^+$ cells with the antibodies α-nestin, α-GalC, α-GFAP, α-β-III tubulin, and α-p75 (p75, an antibody against the low-affinity NGF receptor, has been described to recognize a subset of astrocytes) showed that none of the lineage markers were coexpressed on A2B5$^+$ cells (Fig. 3). A substantial subset of the A2B5$^+$ cells, however, expressed α-nestin. This coexpression of α-nestin and A2B5 has previously been described on O-2A progenitor cells. Thus NEP-derived A2B5$^+$ cells are antigenically similar to O-2A progenitor cells.

A2B5$^+$ cells when stained after an additional 2 days in

TABLE 1
Antibodies Which Identify Specific Cell Types

Antibody/dilution			Antigen recognized	Cell type recognized
A2B5[a]	Mouse (IgM)	1:2	Gangliosides	Glial precursors
O4	Mouse (IgM)	1:2	Galactoside	Oligodendrocytes/precursors
α-GalC	Mouse (IgG)	1:2	Galactocerebroside	Oligodendrocytes
α-GFAP	Rabbit (IgG)	1:500	Glial fibrillary acid protein	Astrocytes
α-β-III tubulin	Mouse (IgG)	1:400	β-III tubulin	Neurons
RT-97	Mouse (IgG)	1:5	Neurofilament	Neurons

Note. Antibodies were used in combination for double or triple label experiments.
[a] A2B5 was originally identified as a neuronal marker (Eisenbarth, 1979). We and others have found that A2B5 is specific for glial cells in this system.

cultures had begun to express glial-specific markers. A subpopulation of cells were clearly GalC$^+$ by that time. To confirm that cells were sequentially differentiating into oligodendrocytes, cultures were stained with O4 and α-GalC. As expected, 30% of the O4$^+$ cells coexpressed α-GalC, resembling immature oligodendrocytes. Double labeling with A2B5 and α-GFAP showed that 10% of the A2B5$^+$ cells were also GFAP$^+$, resembling the antigenic characteristic of type-2 astrocytes (Figs. 4A–4F). All the markers which were coexpressed at that later time point on a subset of A2B5$^+$ cells are characteristic of cells which have been described to belong to the O-2A lineage. This observation suggested that at least a subset of the A2B5$^+$ cells represented glial precursor cells and that A2B5 was a useful marker to define this subpopulation of cells in more detail.

A2B5$^+$ Cells Arise from Multipotential Stem Cells

To investigate whether A2B5$^+$ cells arise from multipotent NEP cells or whether A2B5$^+$ cells arise from an already committed subpopulation of A2B5$^-$ NEP cells, we plated cells at clonal densities and followed their development in culture for 10 days. Dishes were then double stained with the antibodies A2B5/α-β-III tubulin or A2B5/α-GFAP (Figs. 5A and 5B, respectively) and 132 clones were analyzed (Table 2). Nearly all 132 analyzed clones consisted of a mixture of A2B5$^+$, GFAP$^+$, and β-III tubulin$^+$ cells. Ninety-one percent of the clones contained cells that were either A2B5$^+$ or α-GFAP$^+$, while 93% of the clones were either A2B5$^+$ or α-β-III tubulin$^+$. None of the analyzed clones consisted only of cells which were A2B5$^+$. It is noteworthy that although at this early stage none of the clones contained GalC$^+$ cells, oligodendrocytes could be identified in clonal cultures and in mass cultures at later stages (12–15 days post-CEE condition).

The clonal analyses suggested that the A2B5$^+$ population arose from a common multipotential A2B5$^-$ precursor cell.

A2B5$^+$ Cells Differ from Neuroepithelial Cells in Their Differentiation Potential

The mass culture and the clonal experiments suggested that the marker A2B5$^+$ defines cells which might represent the precursors for glial cells but not for neurons. In order to determine directly whether NEP-derived A2B5$^+$ cells can only give rise to glial cells, we purified the A2B5$^+$ population by immunopanning (see Materials and Methods). After 5 days of culturing NEP cells in the absence of CEE, cells were immunopurified, plated on fibronectin/laminin-coated dishes, and exposed to cytokines previously associated with differentiation of precursor into oligodendrocytes, astrocytes, or neurons. The A2B5 panned population was >98% positive for A2B5$^+$ cells when stained 1 hr after panning. Staining 24 hr after plating showed that all cells of the panned population were A2B5$^+$ and did not express any other lineage markers tested (Fig. 6, α-β-III tubulin staining not shown).

Panned cultures, in the presence of bFGF alone for 5 days consisted of 1% oligodendrocytes, 50% GFAP$^+$ astrocytes, and 49% A2B5$^+$ cells. The proportion of differentiated cells was significantly shifted when the bFGF containing medium was replaced after 3 days with medium supplemented only with PDGF. In this condition, 30% of the culture consisted of oligodendrocytes, 50% of astrocytes, and 20% of the cells were A2B5$^+$ cells (Fig. 7).

Although growth in the presence of bFGF alone was sufficient to allow differentiation of NEP cells into neurons in the parent population (details of the parent population described in Kalyani *et al.*, 1997) we failed to detect any neurons in the A2B5$^+$-panned population cultured in the presence of bFGF (Fig. 7). To enhance the probability of neuronal differentiation, we additionally supplemented the medium with retinoic acid (in this culture condition 10 of 10 clones of the parent population contained β-III tubulin$^+$ neurons). Even in this neuron promoting environment the immunopurified A2B5$^+$ population did not contain β-III tubulin$^+$ cells. It was unlikely that we lost the neuronal population through selective cell death because we did not observe significant cell death in the panned mass cultures at any time, suggesting that neurons did not appear rapidly and died, nor did we detect any evidence of β-III tubulin$^+$ "ghosts."

These results suggested that the precursor cells which are responsible for generating neurons were not part of the

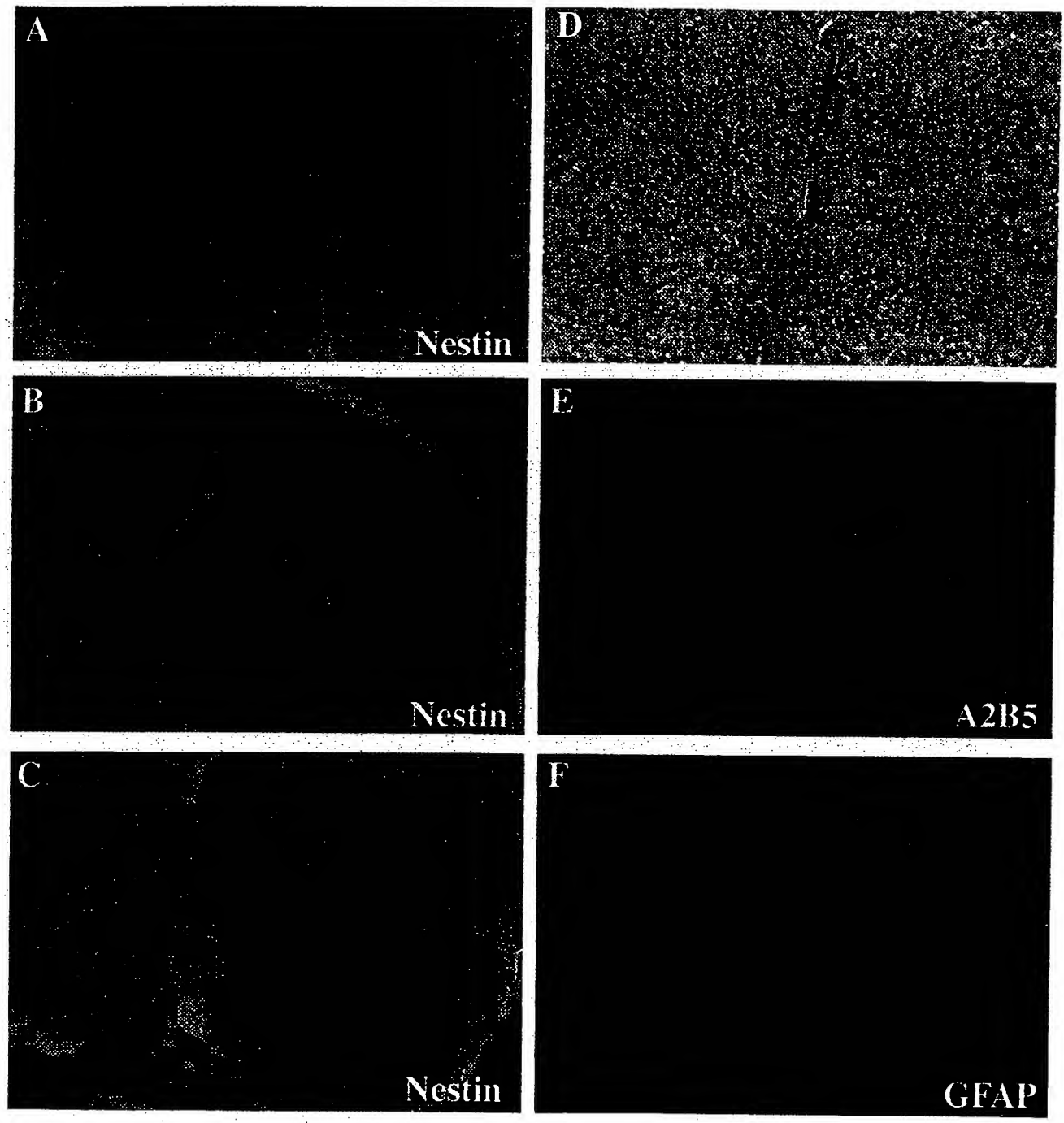

FIG. 1. Neural tube from rat embryos at 10.5 days gestation did not express glial lineage markers. E10.5 rat embryos were collected and neural tubes dissected, fixed, and sectioned. Sections were double labeled with α-nestin, α-GFAP, or A2B5 (A–F). A (fluorescence) and D (phase) show a representative section illustrating nestin expression in the neural tube. B, C, E, F show that nestin$^+$ cells are A2B5$^-$ and GFAP$^-$.

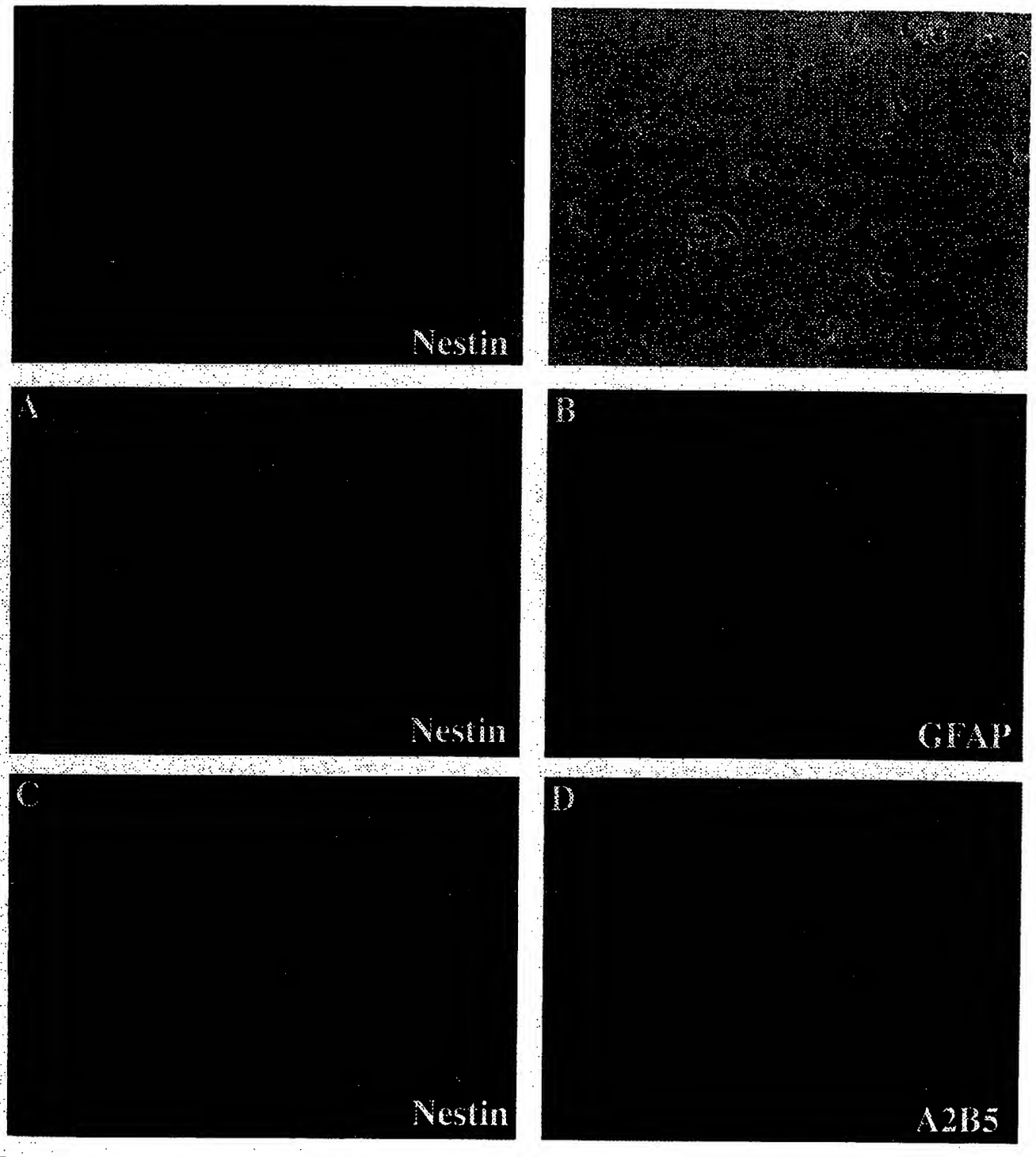

FIG. 2. NEP cells do not express glial markers *in vitro* in the presence of CEE. E10.5 rat neural tube cells were dissociated, plated at low density, and grown in the presence of bFGF and CEE for 5 days. Cells were double labeled with α-nestin and α-GFAP (A and B) or α-nestin and A2B5 (C and D). All NEP cells are nestin$^+$ and GFAP$^-$/A2B5$^-$.

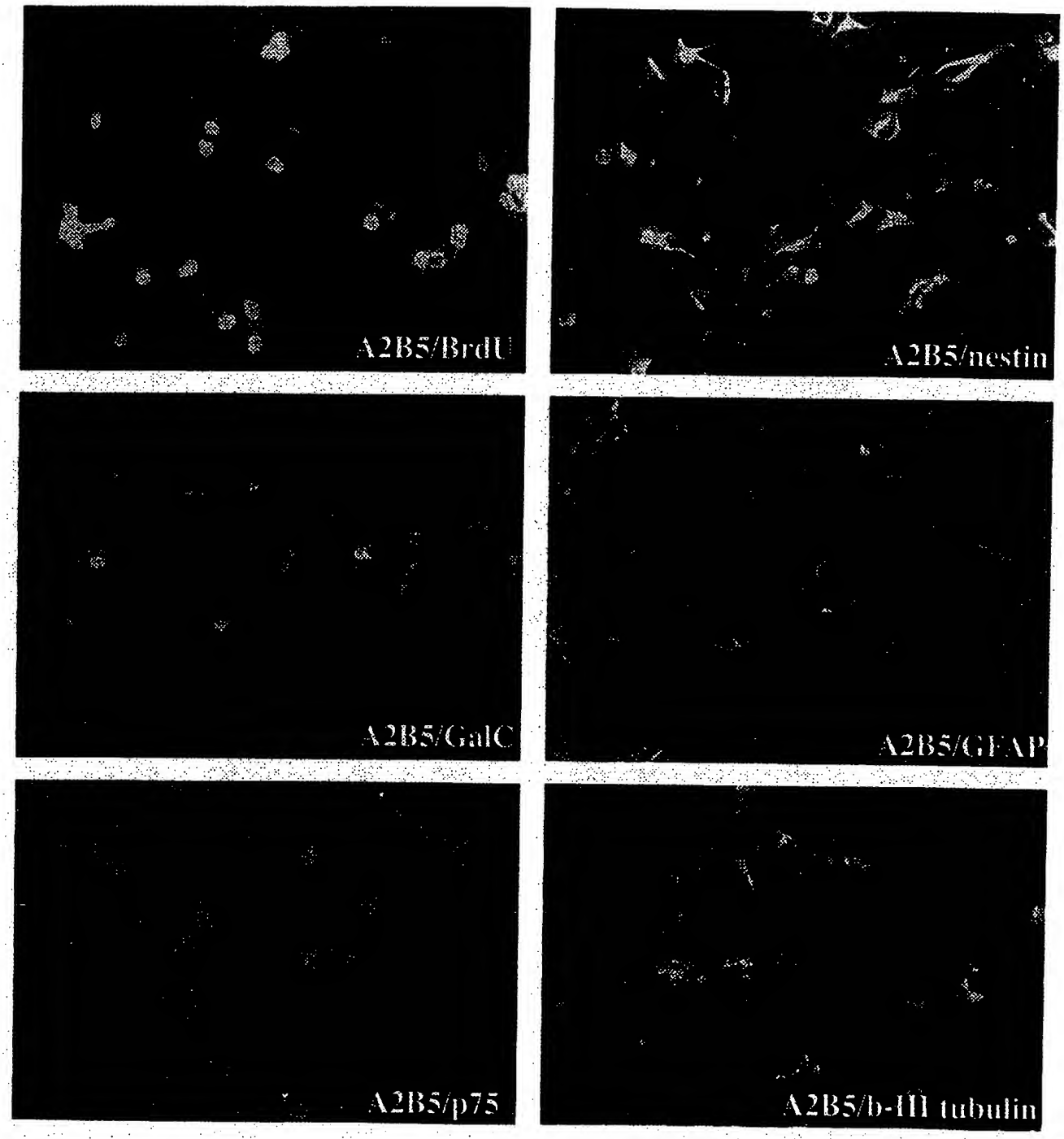

FIG. 3. A2B5$^+$ cells divide *in vitro* and do not express other differentiation markers after 5 days in culture. E10.5 rat neural tube cells were dissociated, grown for 3 days in the presence of CEE and bFGF, and then replated at 5000 cells/coverslip in medium devoid of CEE for an additional 5 days. Cells were incubated for 24 hr with BrDU and stained with anti-BrdU. Parallel cultures were double stained after 7 days with A2B5/α-nestin, A2B5/α-GalC, A2B5/α-GFAP, A2B5/α-p75, or A2B5/α-β-III tubulin. 20% of the A2B5$^+$ cells were nestin$^+$ while all A2B5$^+$ cells were negative for differentiation markers. A2B5 staining is shown in green (fluorescein) while α-BrDU, α-nestin, α-GalC, α-GFAP, α-p75, and α-β-III tubulin are stained in red (rhodamine).

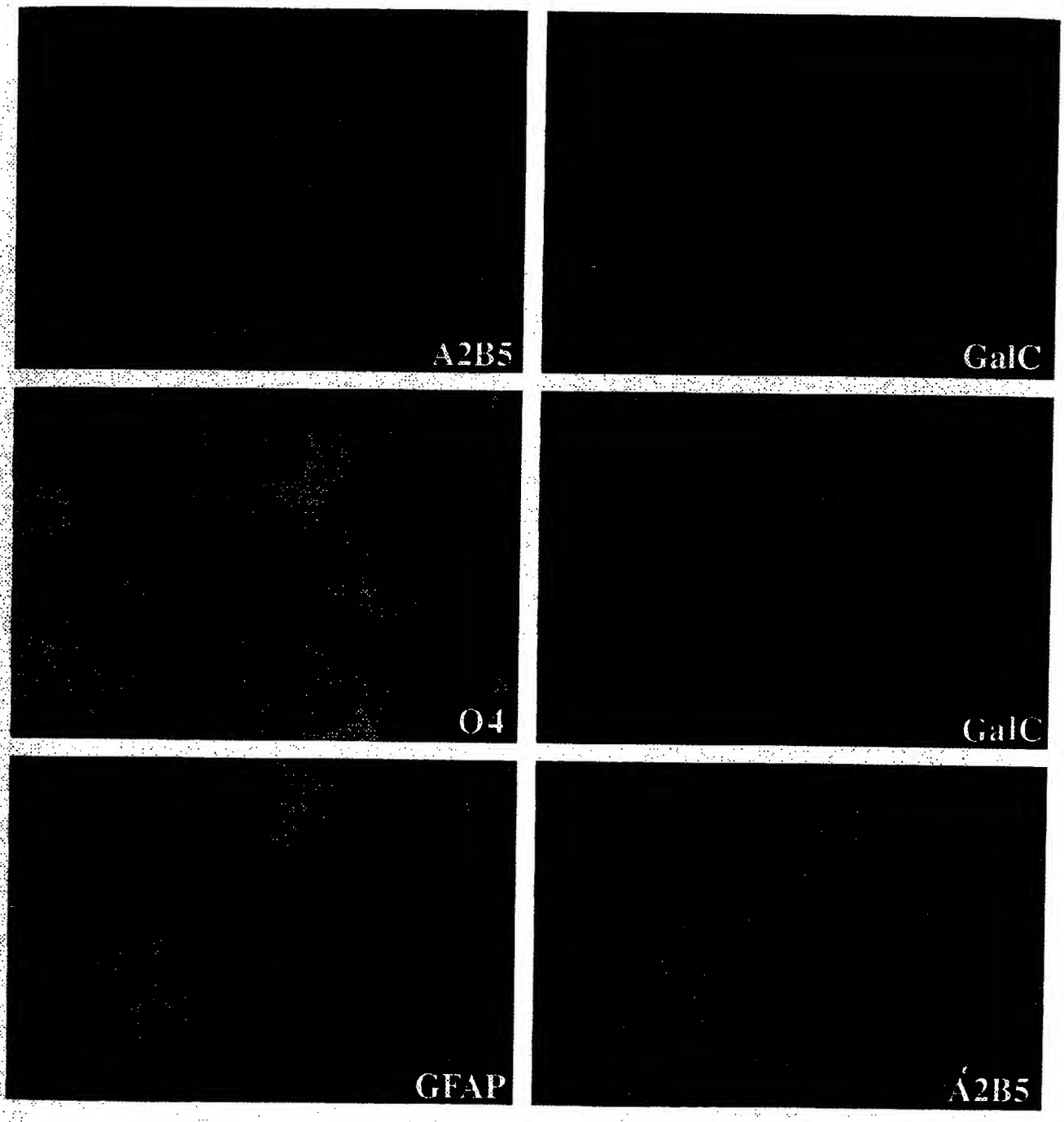

FIG. 4. A2B5 is coexpressed with other glial markers after 7 days in culture. E10.5 NEP cells grown in the presence of bFGF + CEE for 3 days were harvested, replated in bFGF containing NEP medium without additional CEE, and grown for an additional 7 days. Culture dishes were double labeled with A2B5/α-GalC, O4/α-GalC, or A2B5/α-GFAP. A subset of the A2B5$^+$ cells were also GalC$^+$ or GFAP$^+$. The GalC$^+$/O4+ cells represented immature oligodendrocytes (Bansal *et al.*, 1989; Gard and Pfeiffer, 1990; Sommer and Schachner, 1981; Warrington and Pfeiffer, 1992).

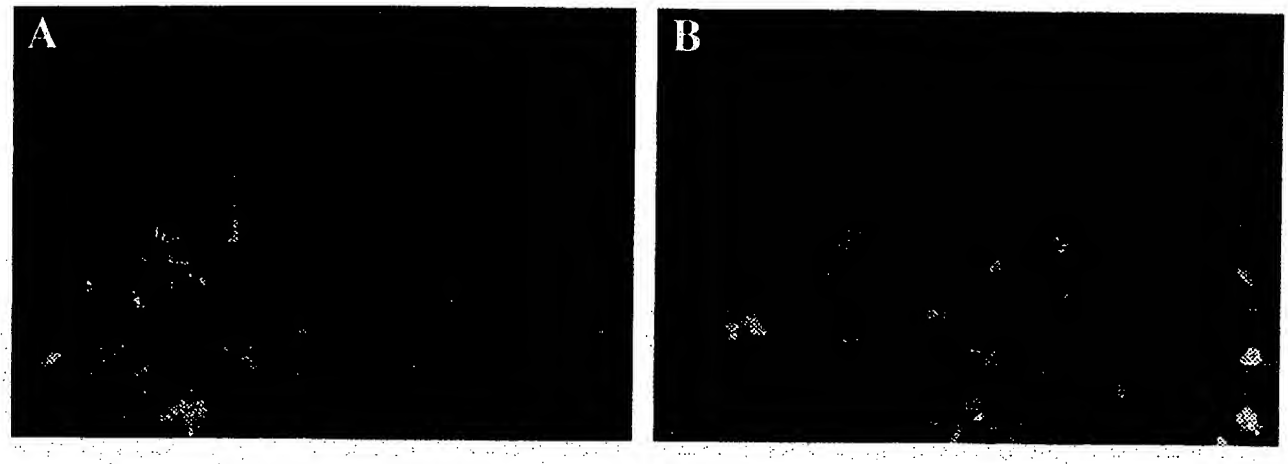

FIG. 5. A2B5$^+$ cells arise from multipotent NEP cells. NEP cells were dissociated and plated at clonal densities. After 5 days clones were stained with A2B5/α-β-III tubulin or A2B5/α-GFAP. Nearly all clones contained A2B5$^+$/β-III tubulin$^+$ cells (A) or A2B5$^+$/GFAP$^+$ cells (B). None of the clones consisted of solely A2B5$^+$ cells. A2B5 staining is shown in green (fluorescein), while α-β-III tubulin and α-GFAP are stained in red (rhodamine).

immunopurified A2B5$^+$ population. As the A2B5-panned cells gave rise to astrocytes and oligodendrocytes but not to neurons, it appeared that the A2B5$^+$ population contained precursor cells which were restricted to the glial lineage.

A2B5$^+$ Cells Generate Multipotential Glial Precursor Cells

Our mass culture experiments suggested that the A2B5 panned population contained cells with a differentiation potential restricted to glial lineages. This experiment, however, did not address whether astrocytes and oligodendrocytes are generated from committed unipotential cells present in the A2B5$^+$ population or whether single cells are bipotential and can generate both astrocytes and oligodendrocytes. To address this question we performed clonal experiments, wherein the A2B5-panned population was stained with A2B5 1 day after panning and cells were plated at limiting dilution in 96-well plates. Wells were scored with immunofluorescence and wells with one A2B5$^+$-stained cell were recorded and cultured in PDGF/bFGF for 7 days. This procedure allowed the expansion of clones and also minimized the amount of cell death occurring when single cells were plated directly into differentiation conditions (unpublished observation). After 7 days expanded clones contained from 50 to 200 cells and were uniformly A2B5$^+$.

TABLE 2
NEP Cells Generate Mixed Clones *in Vitro*

Antigen expressed	Percentage of clones	Number of clones
A2B5$^+$/β-III tubulin$^+$	93	71/76
A2B5$^+$/GFAP$^+$	91	51/56
A2B5$^+$ alone	0	0/132
Total number of clones analyzed	132	

Note. NEP cells were isolated and grown at clonal densities in the absence of CEE and in the presence of bFGF. After 7 days clones were stained with antibodies against A2B5/α-β-III tubulin or A2B5/α-GFAP. The majority of clones contained a mixture of cells either A2B5/β-III tubulin$^+$ or A2B5/GFAP$^+$. No pure clones were found.

The majority of the clones (51) were first washed with bFGF-free DMEM-BS and then switched to PDGF-supplemented medium, an effective culture condition to induce oligodendrocyte generation as shown in mass culture experiments. All clones contained oligodendrocytes, GFAP$^+$ astrocytes, and A2B5$^+$ cells, while none of the clones contained β-III tubulin$^+$ cells, suggesting that single A2B5$^+$ cells were at least bipotential and also were restricted to glial cell lineages (Table 3). A representative clone expanded in PDGF/bFGF, switched to PDGF, and stained after 7 days is shown in Fig. 8.

We also tested the differentiation potential of A2B5$^+$ cells in a culture medium supplemented with bFGF and CNTF. From our panned mass culture experiments, it seemed clear that bFGF alone leads to an increase in the number of GFAP$^+$ astrocytes and a decrease in the number of oligodendrocytes. Depending on culture conditions, CNTF has been shown to promote oligodendrocyte generation (Mayer *et al.*, 1994) or to lead to the generation of type-2 astrocytes, which express A2B5 and GFAP transiently (Lillien and Raff, 1990) or stably (Fok-Seang and Miller, 1992; Madarasz *et al.*, 1991). We analyzed a total of six clones, which were ex-

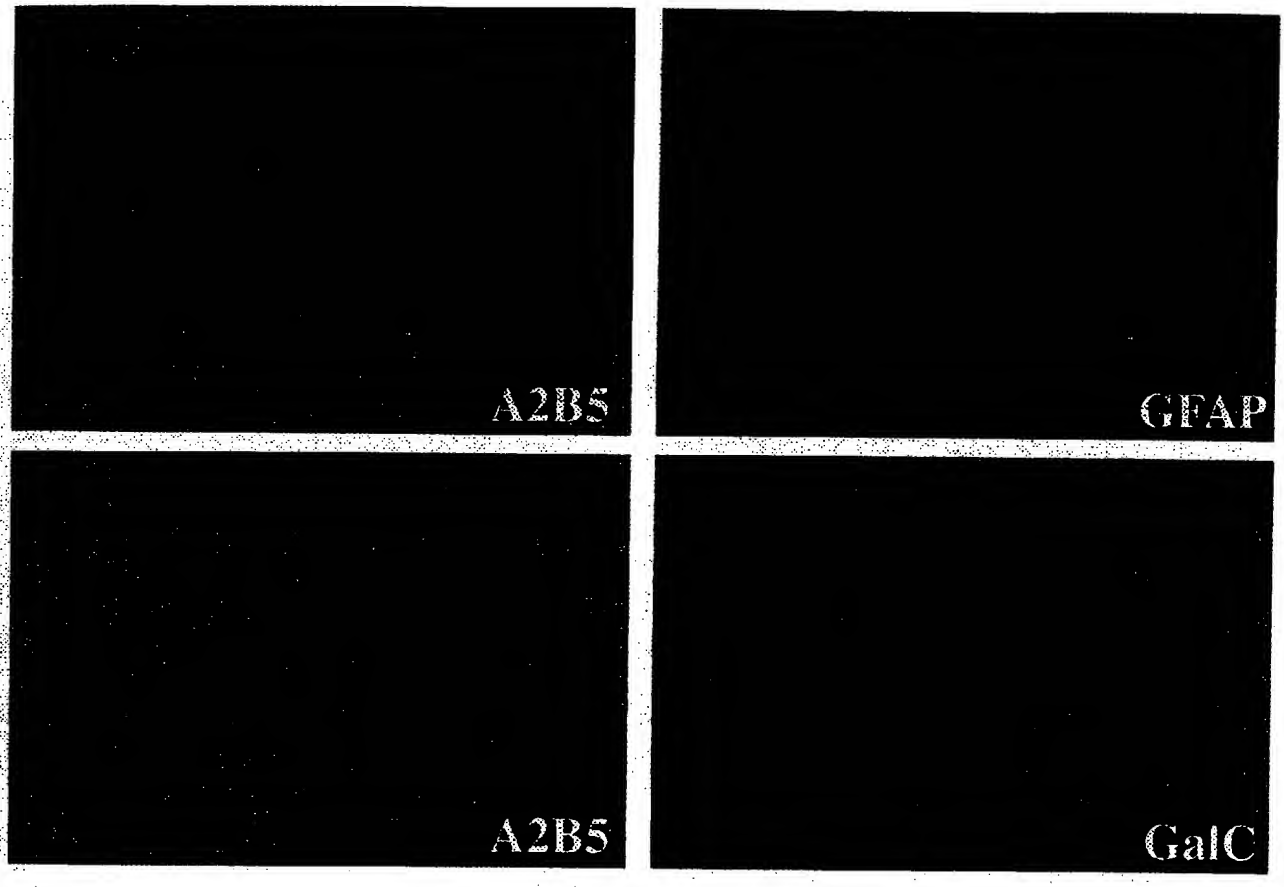

FIG. 6. Panned A2B5$^+$ cells are negative for lineage markers after 1 day is culture. A2B5$^+$ cells were isolated from mixed NEP cells by immunopurification and cultured in the presence of bFGF. Cells were stained after 24 hr with A2B5, α-GFAP, or α-GalC. After 1 day in culture all A2B5$^+$ cells were and α-GFAP$^-$/α-GalC$-$.

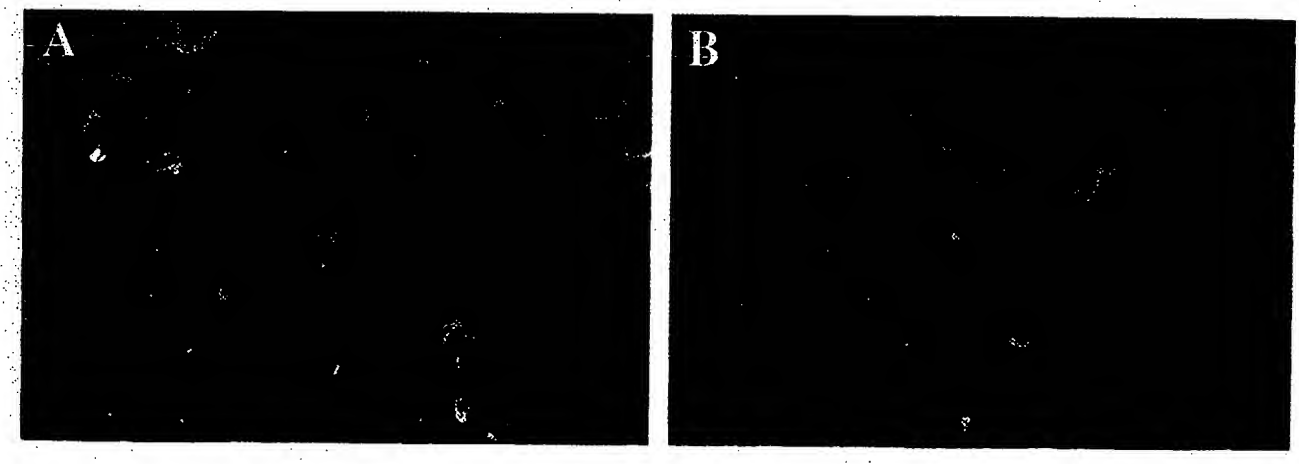

FIG. 7. A2B5$^+$ cells differentiate into oligodendrocytes and astrocytes but not into neurons. A2B5-panned cells were grown on coverslips in the presence of PDGF (A) or bFGF (B) for 9 days. Cultures were then stained with A2B5 (coumarin), α-GalC (rhodamine), α-GFAP (fluorescein), and α-β-III tubulin (rhodamin). Cultures contained oligodendrocytes, astrocytes, and A2B5$^+$ cells but no neurons.

TABLE 3
Individual A2B5$^+$ Cells Are Multipotential

Marker expressed	Growth condition: PDGF	Growth condition: FGF CNTF
A2B5$^+$/GFAP$^+$	0	6
A2B5$^+$	51	6
GFAP$^+$	51	4
GalC$^+$	51	1
β-III tubulin$^+$	0	0
Total number of clones	51	6

Note. NEP-derived A2B5$^+$ cells were immunopurified and plated at serial dilution in 96-well plates. Individual A2B5 immunoreactive cells were identified and expanded in PDGF/bFGF for 7 days, before replacing the culture medium with DMEM-BS supplemented with PDGF or bFGF/CNTF. After 7 days clones were stained. All clones were labeled with all antibodies in one staining procedure (see Material and Methods).

panded in PDGF/bFGF and then switched to bFGF/CNTF. Surprisingly, all six clones contained cells which were A2B5$^+$/GFAP$^+$, resembling the type-2 astrocyte phenotype. Only 1 clone contained GalC$^+$ oligodendrocytes and no clone contained β-III tubulin$^+$ cells (Table 3). This result suggested that in the presence of CNTF and bFGF, A2B5$^+$ cells predominantly differentiate into cells with a type-2 astrocytic phenotype.

We also analyzed five A2B5$^+$ clones in different neuron promoting conditions and, as before, were unable to generate neurons. Five PDGF/bFGF expanded clones were trypsinized, divided into two portions, and replated into either bFGF alone or bFGF supplemented with retinoic acid. Clones were stained with the antibodies A2B5, α-GFAP, α-GalC, and α-β-III tubulin (Table 4). None of the clones, regardless of whether cells were grown in bFGF alone or bFGF/RA, contained β-III tubulin$^+$. In contrast, all five clones consisted of a mixture of cells that were either A2B5$^+$ or GFAP$^+$, but not both. Only one clone grown in bFGF alone contained GalC$^+$ oligodendrocytes, while in bFGF/RA no GalC$^+$ oligodendrocytes were found. These data support the initial observation, that A2B5$^+$ cells isolated from induced NEP cell cultures were multipotential and restricted in their differentiation potential to cells of the glial lineages.

A2B5$^+$ Cells Have an Extended Self-Renewal Potential

In order to fulfill the criteria of a true intermediate precursor, cells need to have an extended self-renewal capacity without losing the ability to differentiate into more than one specific cell type.

To test the self-renewal capacity of individual A2B5$^+$ cells, we selected two clones expanded in PDGF/bFGF for 7 days for long-term culture and passaging. The two clones were refed every other day with PDGF/bFGF and maintained for a total of 3 months with 4 serial passages. Clones were grown in PDGF/bFGF as this combination of cytokines apparently inhibited differentiation and promoted division. Cells were stained before and after each passage and were negative for differentiation markers but A2B5$^+$ at all time points. To determine the differentiation potential of long term clones, during each passage single cells were replated, reexpanded to 50–200 cells, and switched to PDGF alone to promote differentiation. In this secondary cultures oligodendrocytes and astrocytes appeared consistently after 8–10 days (Fig. 9). The ability to differentiate into oligodendrocytes and astrocytes was not altered significantly with increased passages, suggesting that these long-term propagated cells were still multipotential.

Our results show that A2B5$^+$ cells that differentiate from multipotent NEP cells can be expanded and propagated as precursor cells. Passaged individual A2B5$^+$ cells self-renew and are able to generate oligodendrocytes, A2B5$^+$, and A2B5$^-$ astrocytes, but not neurons. NEP-derived A2B5$^+$ cells thus represent multipotential intermediate precursor cells restricted to glial lineages.

DISCUSSION

We have shown that multipotent NEP cells can be induced to generate self-renewing precursor cells restricted to subsequent glial differentiation. This self-renewing precursor population can be isolated by immunopanning using the monoclonal antibody A2B5 and can be maintained in an undifferentiated state over multiple divisions when grown in PDGF and bFGF. A2B5$^+$ cells differ from the parental NEP cell population in antigenic phenotype and differentiation potential. A2B5$^+$ cells lack the ability to differentiate into neurons under conditions that promote neuronal differentiation in NEP cells. A2B5$^+$ cells retain, however, the ability to differentiate into oligodendrocytes and astrocytes and are thus identified as multipotential precursors (Fig. 10).

Several lines of evidence show that the A2B5 immunoreactive glial-restricted precursors arise from multipotent NEP cells; (i) NEP cells are a homogeneously nestin$^+$/A2B5$^-$ population of cells; (ii) Clonal analysis of NEP cell cultures fail to reveal clones that give rise only to glial cells; (iii) A2B5$^+$ cells always arise in clones that contain A2B5$^-$ neurons and astrocytes. Thus we can find no evidence that the NEP cell population contains a committed A2B5$^-$ O2A progenitor. A glial-restricted A2B5$^-$ precursor cells ("pre-O2A") has been described by (Grinspan *et al.*, 1990). This "pre-O2A" precursor, however, can be distinguished from NEP cells in its proliferation response to PDGF and its inability to differentiate into neurons. We suggest that this A2B5$^-$ cell type could represent an additional intermediate

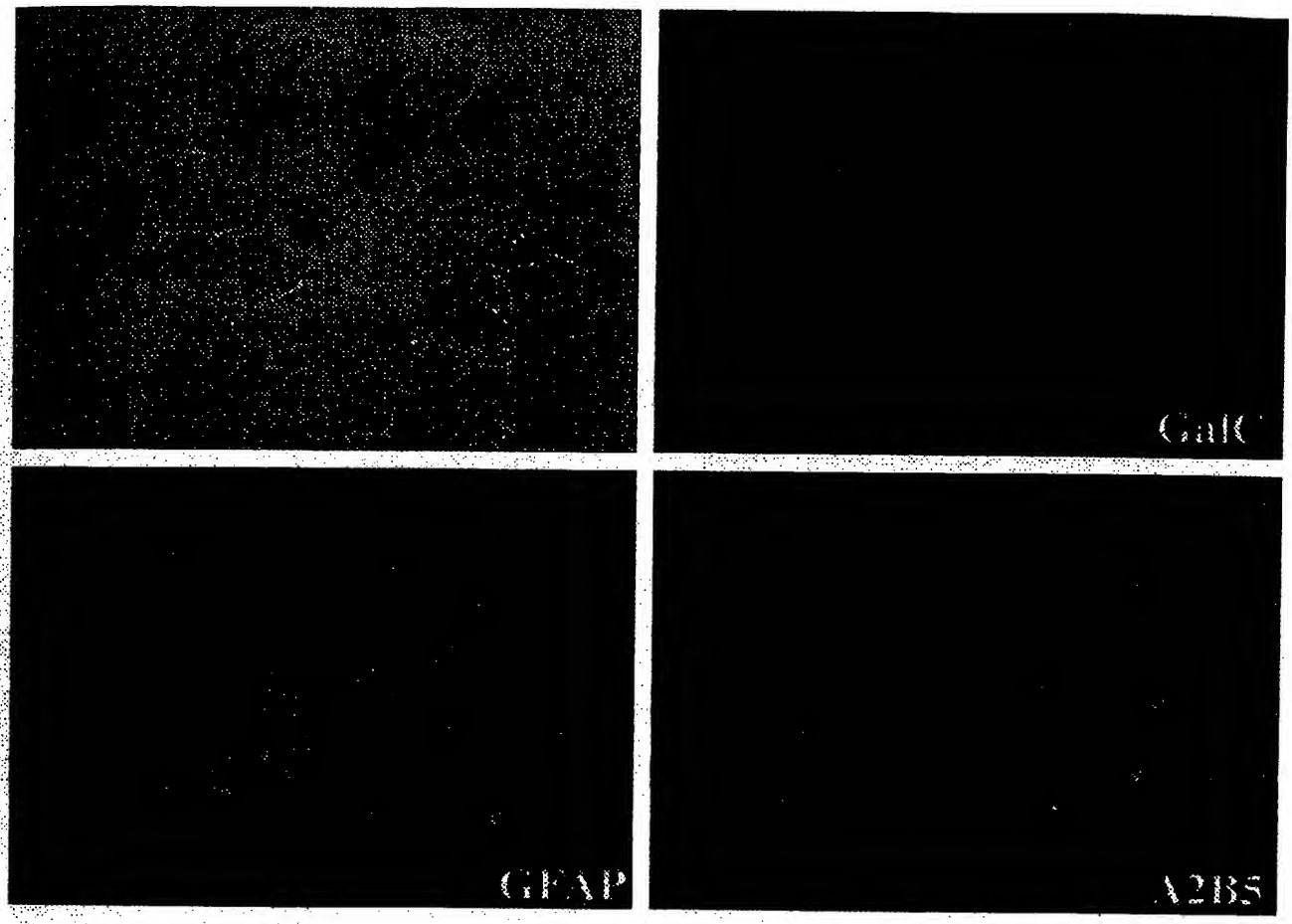

FIG. 8. A2B5$^+$ cells are multipotential. Shown is a representative clone established from single A2B5$^+$ cells. The cell was expanded in PDGF/bFGF to a clonal size of 50–200 cells before the culture was washed with bFGF-free DMEM-BS and further grown in medium supplemented with PDGF. After 8 days clones were stained with A2B5 (rhodamine), α-GalC (coumarin), α-GFAP (fluorescein), and α-β-III tubulin (coumarin). A total of 51 clones was analyzed (Table 3).

step involved in the transition from NEP cells to A2B5$^+$ cells.

The possibility that NEP cells contain subpopulations which are A2B5$^-$ that give rise to precursor populations with other kinds of restrictions cannot be ruled out by our present experiments. Retroviral lineage tracing experiments have suggested the existence of oligodendrocyte-neuron precursor cells in addition to neuron-astrocyte or oligodendrocyte-astrocyte precursor cells (Price *et al.*, 1991, 1992; Williams, 1995; Williams *et al.*, 1991). While we cannot identify putative oligodendrocyte-neuron precursor cells in the A2B5$^+$ population, such cells may well exist in our culture conditions among the A2B5$^-$ population. We did indeed observe the generation of oligodendrocytes and neurons when the A2B5$^-$ supernatant from the immunopanning procedure was cultured. These differentiated cells, however, could also have been the progeny of undifferentiated NEP cells. Without additional markers for either NEP cells or A2B5$^-$ oligodendrocyte-neuron progenitor cells this issue cannot be resolved yet. Alternatively it is possible that the oligodendrocyte-neuron precursor cells previously described are multipotent NEP cells that have the capacity to produce all three cell types (oligodendrocytes, neurons, and astrocytes) but do not differentiate into astrocytes due to environmental restrictions and therefore appear to be restricted to an oligodendrocyte-neuron pathway. In addition a true oligodendrocyte-neuron restricted precursor cell could represent a specialized precursor cell pool restricted to the ventricular zone of the cortex.

An important characteristic of the NEP-derived A2B5$^+$ population is the inability to generate neurons under condition where the parent population generates a large number of neurons. For example, 10 of 10 clones of the parent population grown in the presence of bFGF/RA contain β-III tubulin$^+$ neurons compared to 0 of 5 clones of the A2B5$^+$ population. It is noteworthy that the A2B5$^+$ cells when

TABLE 4
Individual A2B5$^+$ Cells Cannot Generate Neurons

Marker expressed	Growth condition: FGF	FGF RA
A2B5$^+$/GFAP$^+$	0	0
A2B5$^+$	5	5
GFAP$^+$	5	5
GalC$^+$	1	0
β-III tubulin$^+$	0	0
Total number of clones	5	5

Note. NEP-derived A2B5$^+$ cells were immunopurified and plated at limiting dilution in 96-well plates. Individual A2B5 immunoreactive cells were identified and expanded in PDGF/bFGF. After 7 days clones were switched to bFGF or bFGF/RA and stained 7 days later. All clones were labeled with the indicated antibodies in one staining procedure (see Materials and Methods).

grown in the presence of retinoic acid (RA) did not only fail to generate neurons, but did also not differentiate into oligodendrocytes. This result is consistent with observations published by Noll and Miller (1994). Although the A2B5$^+$ cell population appears morphologically homogenous and uniform in its antigenic phenotype, it nevertheless remains to be determined whether this population is also truly homogenous in its differentiation potential. All A2B5$^+$ cells that could be clonally expanded in the presence of PDGF and bFGF were multipotential and gave rise to both astrocytes and oligodendrocytes. Whether unipotent cells exist that require other expansion conditions remains a possibility. We note, however, that less than 10% of the A2B5 population underwent cell death when cells were expanded in PDGF and bFGF. Thus, if additional glial restricted precursors exist they likely represent a small fraction of the A2B5 population.

Our clonal analysis of A2B5 immunoreactive cells shows that single A2B5$^+$ cells can be expanded and propagated in a mixture of PDGF/bFGF without losing their differentiation potential. Expanded clones can differentiate into astrocytes and oligodendrocytes but not into neurons. The culture condition determines what glial differentiation pathways are chosen by a clone. PDGF promotes the differentiation into oligodendrocytes, whereas bFGF promotes astrocytic differentiation. The presence of CNTF leads to the coexpression of A2B5 and GFAP in the majority of cells. This appearance of the type-2 astrocytic phenotype could resemble a preference for a specific astrocytic differentiation pathway promoted by CNTF. Whether A2B5$^+$ astrocytes generated in the presence of CNTF represent a functionally distinct phenotype from the A2B5$^-$ astrocytes generated in the presence of bFGF remains to be determined.

Comparison of the NEP-derived A2B5$^+$ precursor with other glial-restricted precursors identified in the CNS (Aloisi *et al.*, 1992; Blau and Hughes, 1990; Cameron and Rakic, 1991; Chan *et al.*, 1990; Cochard and Giess, 1995; Davis and Temple, 1994; Elder *et al.*, 1988; Fok-Seang and Miller, 1994; Fulton *et al.*, 1992; Galileo *et al.*, 1990; Gard *et al.*, 1995; Grinspan *et al.*, 1990; Hardy and Reynolds, 1991; Hardy and V. L. Friedrich, 1996; Knapp, 1991; Luskin *et al.*, 1993; Miller, 1996; Ono *et al.*, 1995; Raff *et al.*, 1983; Rivkin *et al.*, 1995; Wood and Williams, 1984) reveals several similarities and differences. NEP-derived A2B5$^+$ cells share several characteristics with optic nerve-derived O-2A progenitor cells, including morphology, migratory nature, responsiveness to PDGF and bFGF, and the ability to generate oligodendrocytes and type-2 astrocytes. In contrast to postnatal O-2A progenitor cells, however, NEP-derived A2B5$^+$ cells can also give rise to astrocytes that are GFAP$^+$/A2B5$^-$ (and therefore not Type 2 astrocytes). Thus, it is possible, that A2B5$^+$ cells purified from NEP cells represent an earlier stage of glial precursor cell development than the A2B5$^+$ O-2A progenitor cells that have been studied so extensively.

Previous studies have also identified the existence of A2B5$^+$ precursor populations in the spinal cord which are able to generate oligodendrocytes and astrocytes (Fok-Seang

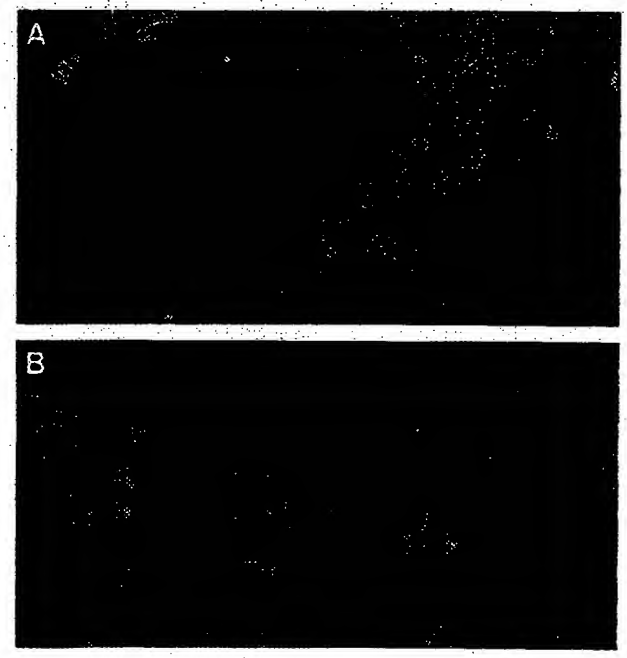

FIG. 9. A2B5$^+$ cells can be propagated without losing their differentiation potential. (A) Phase contrast picture of a long-term clone grown in PDGF/bFGF for 3 months after four passages. (B) An aliquot of this clone was replated after trypsinization on a coverslip, grown in medium supplemented with PDGF, and stained after 10 days with A2B5 (coumarin), α-GFAP (fluorescein), α-GalC (rhodamine), and α-β-III tubulin (rhodamine). The clone contained a mixed population of glial cells but no neurons.

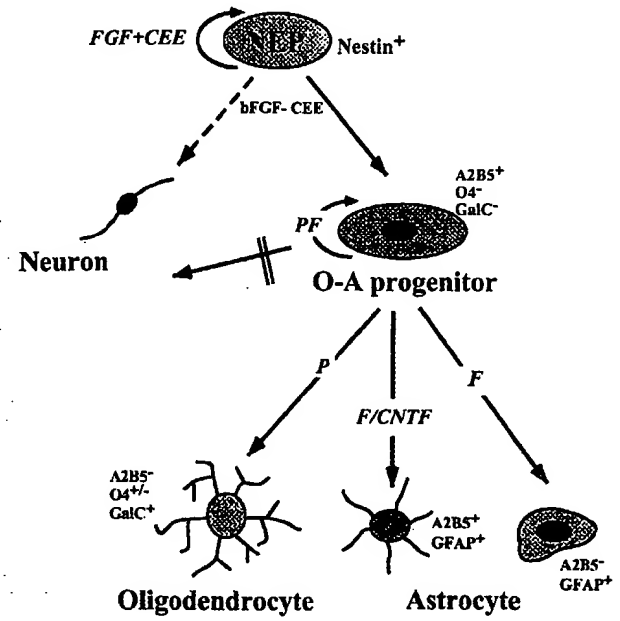

FIG. 10. Model for the generation of glial cells from multipotent NEP cells. While neuroepithelial cells (NEP), which can be expanded in the presence of FGF and chick extract (CEE) are not restricted in their differentiation potential to specific lineages upon CEE withdrawal, A2B5$^+$ cells which arise from this NEP cell population progress to a stage where they lose the potential to generate neurons, but are still able to give rise to oligodendrocytes (O), a pathway promoted by platelet-derived growth factor (P), and antigenically different types of astrocytes (A) depending on the presence of CNTF. These O-A progenitor cells can be expanded in the presence of a combination of P and F.

and Miller, 1992, 1994; Warf *et al.*, 1991). Our results confirm and extend these observations by demonstrating the transition from undifferentiated NEP to at least bipotential A2B5$^+$ cells. It seems likely that at least a subpopulation of NEP-derived A2B5$^+$ cells described here is identical to previously described A2B5$^+$ cells present in embryonic spinal cord.

In summary, we have provided direct evidence for a lineage relationship between multipotent and lineage-restricted precursor cell populations and have identified morphological, antigenic, and cytokine dependence data to distinguish between the two populations. The data presented show for the first time that the transition of multipotent cells to terminally differentiated cells involves the generation of more restricted precursor cells. Equally importantly we have established an accessible culture system to follow the development of isolated precursor cells and to study the cellular and molecular events that regulate differentiation processes. It remains to be determined whether this paradigm is used by early stem cell populations derived from regions other than the spinal cord.

REFERENCES

Aloisi, F., Giampaolo, A., Russo, G., Peschle, C., and Levi, G. (1992). Developmental appearance, antigenic profile, and proliferation of glial cells of the human embryonic spinal cord: An immunocytochemical study using dissociated cultured cells. *Glia* **5**, 171–181.

Bansal, R., Washington, A. E., Gard, A. L., and Pfeiffer, S. E. (1989). Multiple and novel specificities of monoclonal antibodies O1, O4 and R-mAb used in the analysis of oligodendrocyte development. *J. Neurosci. Res.* **24**, 548–557.

Bignami, A., Eng, L. F., Dahl, D., and Uyeda, C. T. (1972). Localization of the glial fibrillary acidic protein in astrocytes, by immunofluorescence. *Brain Res.* **43**, 429–435.

Blau, H. M., and Hughes, S. M. (1990). Cell lineage in vertebrate development. *Curr. Biol.* **2**, 981–985.

Bogler, O., Wren, D., Barnett, S. C., Land, H., and Noble, M. (1990). Cooperation between two growth factors promotes extended self-renewal and inhibits differentiation of oligodendrocyte-type-2 astrocytes (O-2A) progenitor cells. *Proc. Natl. Acad. Sci. USA* **87**, 6368–6372.

Bottenstein, J. E., and Sato, G. H. (1979). Growth of a rat neuroblastoma cell line in serum-free supplemented medium. *Proc. Natl. Acad. Sci. USA* **76**, 514–517.

Cameron, R. S., and Rakic, P. (1991). Glial cell lineage in the cerebral cortex:A review and synthesis. *Glia* **4**, 124–137.

Chan, C. L., Wigley, C. B., and Berry, M. (1990). Oligodendrocyte-type 2 astrocyte (O-2A) progenitor cells from neonatal and adult rat optic nerve differ in their responsiveness to platelet-derived growth factor. *Brain Res. Dev. Brain Res.* **55**, 275–282.

Cochard, P., and Giess, M. C. (1995). [Oligodendrocyte lineage]. *C. R. Seances Soc. Biol. Fil.* **189**, 263–269.

Dahlstrand, J., Collins, V. P., and Lendahl, U. (1992a). Expression of the class VI intermediate filament nestin in human central nervous system. *Cancer Res.* **52**, 5334–5341.

Dahlstrand, J., Zimmerman, L. B., McKay, R. D., and Lendahl, U. (1992b). Characterization of the human nestin gene reveals a close evolutionary relationship to neurofilaments. *J. Cell Sci.* **103**, 589–597.

Davis, A. A., and Temple, S. (1994). A self-renewing multipotential stem cell in embryonic rat cerebal cortex. *Nature* **362**, 363–372.

Eisenbarth, G. S., Walsh, F. S., and Nirenberg, M. (1979). Monoclonal antibody to plasma membrane antigen of neurons. *Proc. Natl. Acad. Sci. USA***76**, 4913–4917.

Elder, G. A., Potts, B. J., and Sawyer, M. (1988). Characterization of glial subpopulations in cultures of the ovine central nervous system. *Glia* **1**, 317–327.

Fok-Seang, J., and Miller, H. R. (1992). Astrocyte precursors in neonatal rat spinal cord cultures. *J. Neurosci.* **12**, 2751–2764.

Fok-Seang, J., and Miller, R. H. (1994). Distribution and Differentiation of A2B5$^+$ glial precursors in the developing rat spinal cord. *J.Neurosci. Res.* **37**, 219–235.

Fulton, B. P., Burne, J. F., and Raff, M. C. (1992). Visualization of O-2A progenitor cells in developing and adult rat optic nerve by quisqualate-stimulated cobalt uptake. *J. Neurosci.* **12**, 4816–4833.

Gage, F. H., Ray, J., and Fisher, L. J. (1995). Isolation, Characterization and Use of Stem Cells from the CNS. *Annu. Rev. Neurosci.* **18**, 159–192.

Galileo, D. S., Gray, G. e., Owens, G. C., Majores, J., and Sanes, J. R. (1990). Neurons and glia arise from a common progenitor in chicken optic tectum: Demonstration with two retroviruses and

cell type-specific antibodies. *Proc. Natl Acad. Sci. USA* **87**, 458–462.

Gard, A. L., and Pfeiffer, S. E. (1990). Two proliferative stages of the oligodendrocyte lineage (A2B5+O4− and O4+GalC−) under different mitogenic control. *Neuron* **5**, 615–625.

Gard, A. L., Williams, W. C. N, and Burrell, M. R. (1995). Oligodendroblasts distinguished from O-2A glial progenitors by surface phenotype (O4+GalC−) and response to cytokines using signal transducer LIFR beta. *Dev. Biol.* **167**, 596–608.

Geisert, E. E., and Frankfurter, A. (1989). The neuronal response to injury as visualized bt immunostaining of class β-tubulin in the rat. *Neurosci. Lett.* **102**, 137–141.

Grinspan, J. B., Stern, J. L., Pustilnik, S. M., and Pleasure, D. (1990). Cerebral white matter contains PDGF-responsive precursors to O-2A cells. *J. Neurosci.* **10**, 1866–1873.

Gritti, A. G., Parati, E. A., Cova, L., Frolichsthal, P., Galli, R., Wanke, E., Faravelli, L., Morassutti, D. J., Roisen, F., Nickel, D., and Vescovi, L. (1996). Multipotential Stem Cells from the Adult Mouse Brain Proliferate and Self-renew in Response to Basic Fibroblast Growth Factor. *J. Neurosci.* **16**, 1091–1100.

Hardy, R., and Reynolds, R. (1991). Proliferation and differentiation potential of rat forebrain oligodendroglial progenitors both in vitro and in vivo. *Development* **111**, 1061–1080.

Hardy, R. J., and V. L. Friedrich, J. (1996). Oligodendrocyte progenitors are generated throughout the embryonic mouse brain, but differentiate in restricted foci. *Development* **122**, 2059–2069.

Kalyani, A., Hobsen C., and Rao M. (1997). Neuroepithelial stem cells from the embryonic spinal cord: Isolation, characterization and clonal analysis. *Dev. Biol., submitted.*

Kilpatrick, T. J., and Bartlett, P. F. (1995). Cloned Multipotential Precursors from the Mouse Cerebrum Require FGF-2, whereas Glial Restricted Precursors are Stimulated with Either FGF-2 or EGF. *J. Neurosci.* **15**, 3653–3661.

Knapp, P. E. (1991). Studies of glial lineage and proliferation in vitro using an early marker for committed oligodendrocytes. *J. Neurosci. Res.* **30**, 336–345.

Lendahl, U., Zimmermann, L. B., and McKay, R. D. G. (1990). CNS stem cells express a new class of intermediate filament protein. *Cell* **60**, 585–595.

Lillien, L. E., and Raff, M. C. (1990). Differentiation signals in the CNS: type-2 astrocyte development in vitro as a model system. *Neuron* **5**,5896–6273.

Luskin, M. B., Parnavelas, J. G., and Barfield, J. A. (1993). Neurons, astrocytes, and oligodendrocytes of the rat cerebral cortex originate from separate progenitor cells: An ultrastructural analysis of clonally related cells. *J. Neurosci.* **13**, 1730–1750.

Madarasz, E., Theodosis, D. T., and Poulain, D. A. (1991). In vitro formation of type-2 astrocytes derived from postnatal rat hypothalamus or cerebral cortex. *Neuroscience* **43**, 211–221.

Marvin, M., and McKay, R. (1992). Multipotential stem cells in the vertebrate CNS. *Semin. Cell Biol.* **3**, 401–411.

Mayer, M., Bhakoo, K., and Noble, M. (1994). Ciliary neurotrophic factor and leukemia inhibitory factor promote the generation, maturation and survival of oligodendrocytes. *Development* **120**, 142–153.

Mayer, M., Bogler, O., and Noble, M. (1993). The inhibition of oligodendrocytic differentiation of O-2A progenitors caused by basic fibroblast growth factor is overridden by astrocytes. *Glia* **8**, 12–19.

McKinnon, R. D., Matsui, T., Dubois-Dalcq, M., and Aaronson, S. A. (1990). FGF modulates the PDGF-driven pathway of oligodendrocytic development. *Neuron* **5**, 603–614.

Miller, R. H. (1996). Oligodendrocyte origins. *Trends Neurosci.* **19**, 92–96.

Morrison, S. J., Shah, N. M., and Anderson, D. J. (1997). Regulatory Mechanism in stem cell biology. *Cell* **88**, 287–298.

Noble, M., Fok Seang, J., Wolswijk, G., and Wren, D. (1990). Development and regeneration in the central nervous system. *Philos. Trans. R. Soc.Lond. B Biol. Sci.* **327**, 127–143.

Noble, M., Murray, K., Stroobant, P., Waterfield, M. D., and Riddle, P. (1988). Platelet-derived growth factor promotes division and motility and inhibits premature differentiation of the oligodendrocyte/type-2 astrocyte progenitor cell. *Nature* **333**, 560–562.

Noll, E., and Miller, R. H. (1993). Oligodendrocyte precursor originate at the ventral ventricular zone dorsal to the ventral midline in the embryonic rat spinal cord. *Development* **118**, 563–573.

Noll, E., and Miller, R. H. (1994). Regulation of oligodendrocyte differentiation: A role for retinoic acid in the spinal cord. *Development* **120**, 649–660.

Ono, K., Bansal, R., Payne, J., Rutishauser, U., and Miller, R. H. (1995). Early development and dispersal of oligodendrocyte precursors in the embryonic chick spinal cord. *Development* **121**, 1743–1754.

Price, J., Turner, D., and Cepko, C. (1987). Lineage analysis in the vertebrate nervous system by retrovirus-mediated gene transfer. *Dev. Biol.* **84**, 156–160.

Price, J., Williams, B., and Grove, E. (1991). Cell lineage in the cerebral cortex. *1991* **2**, 23–28.

Price, J., Williams, B., and Grove, E. (1992). The generation of cellular diversity in the cerebral cortex. *Brain Pathol.* **2**, 23–29.

Pringle, N. P., and Richardson, W. D. (1993). A singularity of PDGF alpha-receptor expression in the dorsoventral axis of the neural tube may define the origin of the oligodendrocyte lineage. *Development* **117**, 525–533.

Raff, M. C., Miller, R. H., and Noble, M. (1983). A glial progenitor cell that develops in vitro into an astrocyte or an oligodendrocyte depending on culture medium. *Nature* **303**, 390–396.

Reynolds, B. A., Tetzlaff, W., and Weiss, S. (1992). A mulitpotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes. *J. Neurosci.* **12**, 4565–4574.

Reynolds, B. A., and Weiss, S. (1996). Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. *Dev. Biol.* **175**, 1–13.

Rivkin, M. J., Flax, J., Mozell, R., Osathanondh, R., Volpe, J. J., and Villa Komaroff, L. (1995). Oligodendroglial development in human fetal cerebrum. *Ann. Neurol.* **38**, 92–101.

Skoff, R. P. (1996). The Lineages of Neuroglial Cells. *Neuroscientist* **2**, 335–344.

Sommer, I., and Schachner, M. (1981). Monoclonal Antibody (O1-O4) to oligodendrocyte cell surfaces: An immunocytological study in the central nervous system. *Dev. Biol.* **83**, 311–327.

Stemple, D. L., and Anderson, D. J. (1992). Isolation of a stem cell for neurons and glia from the mammalian neural crest. *Cell* **71**, 973–985.

Stemple, D. L., and Mahanthappa, N. K. (1997). Neural Stem cells are blasting off. *Neuron* **18**, 1–4.

Stemple, D. L., Mahanthappa, N. K., and Anderson, D. J. (1988). Basic FGF induces neuronal differentiation, cell division, and NGF dependence in chromaffin cells: A sequence of events in sympathetic development. *Neuron* **1**, 517–525.

Tempel, S., and Raff, M. C. (1986). Clonal Analysis of Oligodendrocyte development in culture: Evidence for a developmental clock that counts cell division. *Cell* **44**, 773–779.

Temple, S., and Qian, X. (1996). Vertebrateneural progenitor cells: Subtypes and regulation. *Curr. Opin. Neurobiol.* **6**, 11–17.

Temple, S., and Raff, M. C. (1985). Differentiation of a bipotential glial progenitor cell in single cell microculture. *Nature* **313**, 223–225.

Tohyama, T., Lee, V. M., Rorke, L. B., Marvin, M., McKay, R. D., and Trojanowski, J. Q. (1992). Nestin expression in embryonic human neurocpithelium and in human neurocpithelial tumor cells. *Lab. Invest.* **66**, 303–313.

Tohyama, T., Lee, V. M., Rorke, L. B., Marvin, M., McKay, R. D., and Trojanowski, J. Q. (1993). Monoclonal antibodies to a rat nestin fusion protein recognize a 220-kDa polypeptide in subsets of fetal and adult human central nervous system neurons and in primitive neuroectodermal tumor cells. *Am. J. Pathol.* **143**, 258–268.

Trimmer, P. A., Phillips, L. L., and Steward, O. (1991). Combination of in situ hybridization and immunocytochemistry to detect messenger RNAs in identified CNS neurons and glia in tissue culture. *J. Histochem. Cytochem.* **39**, 891–898.

Warf, B. C., Fok-Seang, J., and Miller, R. H. (1991). Evidence for the ventral origin of oligodendrocyte precursors in the rat spinal cord. *J. Neurosci.* **11**, 2477–2488.

Warrington, A. E., and Pfeiffer, S. E. (1992). Proliferation and differentiation of O4$^+$ oligodendrocytes in postnatal rat cerebellum: Analysis in unfixed tissue slices using anti-glycolipid antibodies. *J. Neurosci. Res.* **33**, 338–353.

Williams, B. (1995). Precursor cell types in the germinal zone of the cerebral cortex. *BioEssays* **17**, 391–393.

Williams, B. P., Read, J., and Price, J. (1991). The generation of neurons and oligodendrocytes from a common precursor cell. *Neuron* **7**, 685–693.

Wood, P. M., and Williams, A. K. (1984). Oligodendrocyte proliferation and CNS myelination in cultures containing dissociated embryonic neuroglia and dorsal root ganglion neurons. *Brain Res.* **314**, 225–241.

Wysocki, L. J., and Sato, V. L. (1978). "Panning" for lymphocytes: A method for cell selection. *Proc. Natl. Acad. Sci. USA* **75**, 2844–2848.

Yokoyama, M., Black, I. B., and Dreyfus, C. F. (1993). NGF increases brain astrocyte number in culture. *Exp. Neurol.* **124**, 377–380.

Yu, W. P., Collarini, E. J., Pringle, N. P., and Richardson, W. D. (1994). Embryonic expression of myelin genes: Evidence for a focal source of oligodendrocyte precursors in the ventricular zone of the neural tube. *Neuron* **12**, 1353–1362.

Zimmerman, L., Parr, B., Lendahl, U., Cunningham, M., McKay, R., Gavin, B., Mann, J., Vassileva, G., and McMahon, A. (1994). Independent regulatory elements in the nestin gene direct transgene expression to neural stem cells or muscle precursors [published erratum appears in Neuron 1994 Jun; 12(6):following 1388]. *Neuron* **12**, 11–24.

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